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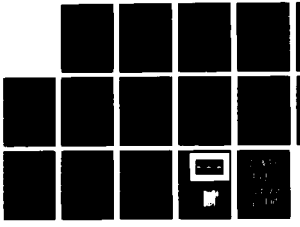

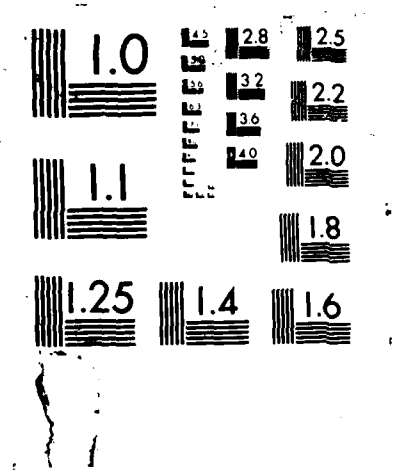
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Transposon Tn916 Mutagenesis in Bacillus anthracis

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ABSTRACT

Mutagenesis of Bacillus anthracis by the streptococcal tetracycline resistance transposon, Tn916, is described. Tn916 was transferred from Streptococcus faecalis strain DS16C1 to B. anthracis VNR-1 by conjugation in a standard filter mating procedure. Tetracycline-resistant (Tc^r) transconjugants were obtained at a frequency of 1.6 X 10^{-8} per donor CFU. When donor and recipient cells were treated with nafcillin prior to conjugation, this frequency was increased nearly tenfold. Nafcillin pretreatment of donor and recipient strains was used in all subsequent conjugation experiments. S. faecalis strain CG110, containing multiple chromosomal insertions of Tn916, transferred the transposon to B. anthracis VNR-1 at a frequency of 9.3 X 10^{-5}. A Tc^r B. anthracis transconjugant, strain VNR-1-tet-1, transferred Tn916 to B. anthracis strain UM23-1 and Bacillus subtilis BST1 at frequencies of 2.1 X 10^{-4} and 5.8 X 10^{-8}, respectively. The transfer of Tn916 occurred only on membrane filters, since no Tc^r transconjugants were obtained when strains VNR-1-tet-1 and UM23-1 were mixed and incubated in broth culture. The presence of the Tn916-associated tetM gene in Tc^r B. anthracis and B. subtilis transconjugants was confirmed in hybridization experiments by using a 5-kilobase-pair DNA fragment containing the tetM gene as a probe. Of 3000 B. anthracis UM23-1 Tc^r transconjugants tested, 21 were phenylalanine auxotrophs, and two were auxotrophic for phenylalanine, tyrosine, and tryptophan.

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Mutagenesis of Bacillus anthracis by the streptococcal tetracycline resistance transposon, Tn916, is described. Tn916 was transferred from Streptococcus faecalis strain DS16C1 to B. anthracis VNR-1 by conjugation in a standard filter mating procedure. Tetracycline-resistant (Tc^r) transconjugants were obtained at a frequency of 1.6×10^{-8} per donor CFU. When donor and recipient cells were treated with nafcillin prior to conjugation, this frequency was increased nearly tenfold. Nafcillin pretreatment of donor and recipient strains was used

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in all subsequent conjugation experiments. S. faecalis strain CG110, containing multiple chromosomal insertions of Tn916, transferred the transposon to B. anthracis VNR-1 at a frequency of 9.3×10^{-5}. A Tc^r B. anthracis transconjugant, strain VNR-1-tet-1, transferred Tn916 to B. anthracis strain UM23-1 and Bacillus subtilis BST1 at frequencies of 2.1×10^{-4} and 5.8×10^{-6}, respectively. The transfer of Tn916 occurred only on membrane filters, since no Tc^r transconjugants were obtained when strains VNR-1-tet-1 and UM23-1 were mixed and incubated in broth culture. The presence of the Tn916-associated tetM gene in Tc^r B. anthracis and B. subtilis transconjugants was confirmed in hybridization experiments by using a 5-kilobase-pair DNA fragment containing the tetM gene as a probe. Of 3000 B. anthracis UM23-1 Tc^r transconjugants tested, 21 were phenylalanine auxotrophs, and two were auxotrophic for phenylalanine, tyrosine, and tryptophan.

INTRODUCTION

An important focus of research on the pathogen, Bacillus anthracis, has been the generation of prototype, live-vaccine strains for immunization against anthrax. Transposon mutagenesis would appear to be a potentially useful tool in this research. Indeed, Koehler and Thorne have transferred Tn917 into B. anthracis. (C. B. Thorne, personal communication.) We therefore undertook the development of a transposon mutagenesis system in B. anthracis. We chose Tn916, a 15-kilobase-pair (kbp), tetracycline resistance (Tc^r) transposon, originally detected in Streptococcus faecalis strain DS16 (9, 10), for this purpose. It seemed a particularly good candidate for two reasons: i) it encodes conjugative functions which facilitate its transfer from one cell to another in the absence of plasmid DNA (9, 10), and ii) it has been shown to insert into different sites on the recipient chromosome, with some transconjugants containing more than one copy of the transposon (11). Tn916 has mediated its own transfer to other strains of S. faecalis, as well as to S. mutans, S. agalactiae, and S. lactis during mixed incubation on membrane filters (5). It has also transferred itself from S. faecalis to Staphylococcus aureus (19). However, to our knowledge, the self-transmission of Tn916 from streptococci into Bacillus species has not been reported, although Christie et al. (4) reported that Tn925, which is similar to Tn916, transferred itself from S. faecalis to Bacillus subtilis.

Our primary goals in this initial work were to develop methodology for Tn916 mutagenesis in B. anthracis and to identify

mutants, specifically mutants deficient in their ability to synthesize aromatic amino acids, which may serve as candidate live vaccines.

(This work was presented in part at the Annual Meeting of the American Society For Microbiology, Washington, D. C., March, 1986, and at the Tenth Annual Mid-Atlantic Extrachromosomal Genetic Elements Meeting, October, 1986, Virginia Beach, Va.)

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in these studies are listed in Table 1. Brain heart infusion (BHI), brain heart infusion agar (BHIA), nutrient broth (NB), nutrient agar (NA), and tryptic soy agar (TSA) were obtained from Difco Laboratories (Detroit, Mich.) and were prepared according to the manufacturer's instructions. Sheep blood agar (SBA) plates contained 5% sheep blood in Bacto blood agar base (Difco). Horse blood agar (HBA) plates contained 5% horse blood in Columbia blood agar base (Difco). The preparation of R agar (RA), which consisted of the defined, synthetic B. anthracis growth medium (R medium) with 1.5 % Bacto agar (Difco) added, has been described previously (23). Plates of Brewer's agar (BA) consisted of the synthetic B. anthracis growth medium described by Brewer et al. (3) with 1.5% agar. For isolation of specific B. anthracis auxotrophic mutants, phenylalanine, tyrosine, and tryptophan, together or independently, were omitted from BA. Antibiotics added to the media were used in the following concentrations: tetracycline, 10 μg/ml; streptomycin, 500 μg/ml; kanamycin, 10 μg/ml; nafcillin, 100 μg/ml.

Conjugation experiments. All incubations were at 37°C. Prior to mixing, donor and recipient cultures were grown with gentle shaking (100 revolutions per min) in 250-ml flasks containing 100 ml of either BHI or BHI with the appropriate antibiotic. Overnight cultures were diluted 1:10 in fresh medium and allowed to incubate an additional 2 h. When nafcillin was used, it was added to donor and recipient cultures after the first hour of incubation.

Donor and recipient cultures, 1 ml each, were mixed and collected on a Ø.45 μm pore-diameter, cellulose acetate membrane (Gelman Sciences, Ann Arbor, Mich.). After washing with 1Ø ml of medium, the filter was incubated, cell-side-up, on an SBA plate for 18 h. Cells were scraped from the filters with a bent glass rod and resuspended in Ø.5 ml of sterile phosphate-buffered saline (PBS). This procedure was repeated, and the cell suspensions were pooled in a sterile tube. Tenfold dilutions (Ø.1 ml of each) were spread on appropriate selective plates which were incubated one to three days in 5% CO_2. Conjugation frequencies were calculated as the number of Tc^r transconjugants divided by the number of donor CFU obtained at the end of the mating.

The procedure used for the broth mating of B. anthracis VNR-1-tet-1 with B. anthracis UM23-1 was a modification of the technique described by Gonzalez et al. (13). Overnight cultures of VNR-1-tet-1 in NB with tetracycline and UM23-1 in NB with streptomycin were each diluted 1:1ØØ into 1Ø ml of fresh NB. After three h incubation at 37°C with gentle shaking, tubes containing 2.Ø ml of nutrient broth were inoculated with 2Ø μl of the donor and recipient cultures. These fresh cultures were incubated 2Ø h with gentle shaking at 37°C, then plated onto NA containing tetracycline and streptomycin to screen for transconjugants. Donor and recipient viabilities were determined by plating onto NA containing tetracycline or streptomycin, respectively.

Isolation of B. anthracis auxotrophic mutants. B. anthracis Tc^r transconjugants (from the mating of strains VNR-1-tet-1 and UM23-1) were inoculated onto BA plus tetracycline plates, which also

contained phenylalanine, tyrosine, and tryptophan, or which lacked one or more of the three amino acids. After incubation at 37°C for 48 h, auxotrophic clones were selected which grew on BA but not on BA lacking the indicated amino acid or amino acids.

Determination of frequency of spontaneous mutation to antibiotic resistance. Bacterial strains were inoculated onto BHIA containing either kanamycin (B. subtilis BST1), tetracycline (B. anthracis VNR-1-tet-1), streptomycin (B. anthracis UM23-1), or no antibiotic (B. anthracis VNR-1). Growth was scraped from each plate and suspended in PBS. Aliquots (Ø.1 ml) were spread onto plates of BHIA containing the appropriate antibiotic as above as well as tetracycline for B. anthracis strains UM23-1 and VNR-1 and B. subtilis BST1, or streptomycin for B. anthracis VNR-1-tet-1. Serial dilutions of other Ø.1-ml aliquots were spread onto plates of TSA for determination of total viability. After incubation for 16 to 2Ø h at 37°C, colony counts were made, and the frequencies of spontaneous mutation to antibiotic resistance were determined.

Purification of DNA. Plasmid DNA was purified from E. coli by a modification of the alkaline lysis methods of Birnboim and Doly (2), as described by Maniatis et al. (22), then ultracentrifugation in CsCl-ethidium bromide gradients. B. subtilis and B. anthracis DNA was prepared according to the sodium dodecyl sulfate-NaOH-heat method of Green et al. (14), except that the cells were incubated with lysozyme (15 mg/ml) for 3Ø min prior to addition of lysis buffer. Similarly, prior to purification of streptococcal whole cell DNA by the procedures of Anderson and McKay (1), the resuspended cells were treated with lysozyme as above. The DNA

preparations were stored at 4°C or -70°C, either in Tris-EDTA buffer (25) or as ethanol-precipitated pellets.

Detection of DNA by hybridization. For slot-blotting procedures, total cell DNA was prepared for hybridization by the procedure described by Kafatos et al. (20). The DNA was denatured by heating in 0.3 M NaOH at 65°C for 30 min. After cooling to room temperature, samples were neutralized by addition of 2.0 M ammonium acetate. For each preparation, approximately 500 ng of DNA (determined by absorbance at 260 nm) in 0.4 ml of 0.9 M sodium chloride-0.09 M sodium citrate solution, pH 7.0 (21), was applied to nitrocellulose membrane strips by using the Minifold Slot Blot System of Schleicher and Schuell (Keene, N.H.). The strips were dried and heated at 70°C for 30 min in a vacuum oven.

The *tetM* DNA probe (16) was prepared by digestion of plasmid pJI3 DNA with *Hin*cII restriction endonuclease (International Biotechnologies, Inc., New Haven, Conn.). A 5-kb *Hin*cII fragment containing the *tetM* gene of Tn*916* was purified from the pACYC177 vector by electrophoretic elution from an agarose gel (22). One microgram of the *tetM* gene fragment was labeled with alpha ^{32}P-deoxycytidine 5'-triphosphate (New England Nuclear Corp., Boston, Mass.) by nick translation. Reagents for the nick translation reaction were obtained from BRL (Gaithersburg, Md.) and were used as recommended by the supplier. Unincorporated nucleotides were removed by filtration over Sephadex G-50 (Sigma Chemical Co., St. Louis, Mo.) minicolumns (26). The nitrocellulose membrane strips containing the samples of total-cell DNA to be probed were placed in a solution containing 50% formamide, 5X Denhardt's solution,

5X SSPE (sodium chloride/sodium phosphate/EDTA), Ø.1% sodium dodecyl sulfate, and 1Ø μg of denatured calf thymus DNA per ml, as described by Maniatis et al. (22). The membranes were prehybridized by incubation overnight at 42°C in a shaking water bath. The solution was removed and the membranes hybridized in 1Ø ml of the prehybridization solution containing heat-denatured, ^{32}P-labeled probe DNA (3 X $1Ø^6$ cpm) and calf thymus DNA (1ØØ μg/ml). The membranes were then incubated at 42°C for 16 to 24 h. After hybridization, the strips were washed and subjected to autoradiography as described previously (17).

For probing with pAM12Ø and pAM12ØLT DNA, one microgram each of total-cell DNA from the B. anthracis strains to be probed was digested with Hind III and analyzed by electrophoresis in Ø.8% agarose gels as described previously (17, 22). The two plasmids (1Ø ng each) and lambda DNA digested with Hind III and EcoR I were included in the gels. The gels were then dried in preparation for direct hybridization by the method of Tsao (28).

One microgram each of lambda DNA and plasmid pAM12Ø DNA, and 25Ø ng of plasmid pAM12ØLT DNA were labeled and prepared for probing as described above for the tetM DNA probe. The agarose gels were prepared, hybridized to labeled lambda DNA and either pAM12Ø or pAM12ØLT DNA, and washed and evaluated by autoradiography as described above for the nitrocellulose membranes and as described previously for agarose gels (17).

RESULTS

Effect of nafcillin on frequency of Tc^r transconjugants in mating S. faecalis DS16C1 with B. anthracis VNR-1. The first two filter mating experiments tested the effect of nafcillin on the frequency of conjugation. Transconjugant frequencies when donor (S. faecalis DS16C1) and recipient (B. anthracis VNR-1) were treated with nafcillin prior to mating were 4.8 X 1Ø$^{-8}$ (Experiment 1) and 1.9 X 1Ø$^{-7}$ (Experiment 2), with a mean value of 1.2 X 1Ø$^{-7}$. Frequencies of Tc^r transconjugants when untreated cells were mated were 1.7 X 1Ø$^{-8}$ (Experiment 1) and 1.5 X 1Ø$^{-8}$ (Experiment 2) with a mean value of 1.6 X 1Ø$^{-8}$. Since nafcillin pretreatment appeared to increase the frequency of conjugation, the antibiotic was used in all further filter matings.

Filter mating. The data shown in Table 2 demonstrate that mating Tn916 donor S. faecalis DS16C1 with B. anthracis VNR-1 resulted in the appearance of Tc^r B. anthracis clones. Furthermore, the mating of one of the transconjugant clones, VNR-1-tet-1, with a Sm^r strain of B. anthracis (UM23-1) and with a kanamycin-resistant (Km^r) strain of B. subtilis (BST1), yielded Tc^r B. anthracis and B. subtilis transconjugants. In all mating experiments the frequencies with which Tc^r transconjugants appeared were similar to those reported for the transfer of Tn916 among streptococci (5). The mean frequency when the two B. anthracis strains were mated was especially high, 2.1 X 1Ø$^{-4}$. This value is similar to the highest reported Tn916 transconjugant frequency of 2.4 X 1Ø$^{-4}$

during matings of donor Streptococcus agalactiae with recipient S. faecalis (5). When S. faecalis CG11Ø was mated with B. anthracis VNR-1, Tc^r transconjugants appeared at a frequency over 1ØØ times higher than that observed when DS16C1 was mated with VNR-1. No Tc^r transconjugants were recovered after mating of B. anthracis strains VNR-1-tet-1 and UM23-1 in broth culture. The data from all of the mating experiments suggested that transfer of Tn916 was responsible for the appearance of the Tc^r B. anthracis and B. subtilis clones.

Mutation to antibiotic resistance. The frequency of spontaneous mutation to tetracycline resistance for B. anthracis strains VNR-1 and UM23-1 and for B. subtilis BST1 was less than $4.Ø \times 1Ø^{-1Ø}$. For B. anthracis VNR-1-tet-1, the frequency of mutation to streptomycin resistance was $3.7 \times 1Ø^{-9}$. It thus appeared unlikely that mutation to antibiotic resistance was responsible for the appearance of Tc^r transconjugant clones after mating.

Auxotrophic mutants. Three thousand transconjugant clones from the mating between B. anthracis strains VNR-1-tet-1 and UM23-1 were tested for auxotrophy. Twenty-one were Phe$^-$ and two were Phe$^-$, Tyr$^-$, and Trp$^-$. No mutants isolated were Tyr$^-$ or Trp$^-$ alone. The finding that different mutants were generated in B. anthracis by using Tn916 mutagenesis suggests that the transposon can insert into multiple sites within the cell DNA.

Hybridization studies. Figure 1 shows the results of probing for the presence of tetM DNA in total cell DNA from Tc^r and Tc^s strains. The Tc^r strains, B. subtilis KT1 (A), S. faecalis DS16C1 (B), and B. anthracis VNR-1-tet-1 (C), all hybridized strongly

with the probe. In contrast, the Tc^s strains B. subtilis BST1 (D), S. faecalis JH2-2 (E), and B. anthracis VNR-1 (F) exhibited no hybridization with the probe. These hybridization data clearly indicate the presence of tetM DNA in the transconjugants and further demonstrate that the presence of Tn916 was responsible for the acquisition of tetracycline resistance.

Total B. anthracis cell DNA from the Tc^s recipient strain VNR-1 and from Tc^r transconjugants isolated in three, independent mating experiments was digested with HindIII, which cuts Tn916 at a single site in the tetracycline resistance (tetM) determinant portion of the transposon, about 5-Kb from one end (6). The fragments were separated by agarose gel electrophoresis and, after drying, the gel was incubated for hybridization with the plasmid, pAM12Ø, which contains the entire Tn916 transposon. Different size fragments, all greater than 5 Kb, from each Tc^r strain whose DNA was tested hybridized to the probes (Fig. 2), confirming several possible insertion sites for Tn916 in the B. anthracis DNA. We saw no hybridization with the probe for the Tc^s parental strain VNR-1 (lane H). Prototrophic strains VNR-1-tet-1 (lane E), VNR-1-tet-2 (lane D), VNR-1-tet-3 (lane C), and VNR-1-tet-4 (lane B), Phe$^-$ strain tet B (lane G), and Phe$^-$, Tyr$^-$, Trp$^-$ strain tet C (lane F) all hybridized with the pAM12Ø robe. None of the strains which were tested hybridized with the pAM12ØLT probe from which Tn916 had been excised (data not shown). For a single insertion of Tn916 in the cell DNA, it would have been expected that Tc^r B. anthracis strains would exhibit two bands on the autoradiogram, one greater than 5 kB, the other greater than

10 kB, depending upon the location of the nearest HindIII site on each end of the transposon. The presence of more than two bands for the Tc^r B. anthracis strains indicated multiple insertions of Tn916 were present in the DNAs.

DISCUSSION

The data presented here provide evidence for i) the transfer of Tn916 from S. faecalis to B. anthracis, and the subsequent transfer of this transposon from B. anthracis to another B. anthracis strain and to B. subtilis; ii) the enhancement of Tn916 transfer from S. faecalis to B. anthracis by the growth of donor and recipient strains in the presence of nafcillin; iii) the insertion of Tn916 into more than one site in the B. anthracis DNA; and iv) the generation of specific mutants by transposon mutagenesis of B. anthracis. The range of hosts for Tn916 is unknown, but the transposon may prove to be an effective mutagenic tool in numerous microbial genera. In this regard, Tn916 was recently transferred from S. faecalis to S. aureus by mating on filter membranes (19). Furthermore, Tn916 has been transferred to Mycoplasma species by transformation with pAM12Ø (8) and by mixed incubation of S. faecalis and M. hominis on solid media (24). In these studies Tn916 was shown to have inserted into the Mycoplasma chromosome at several locations. Gawron-Burke and Clewell (12) noted expression of tetracycline resistance in Escherichia coli after transformation with a pBR322-derived plasmid, pGL1Ø1, into which Tn916 had been ligated.

Nafcillin is a synthetic penicillin clinically used against microorganisms which are resistant to penicillin due to the production of beta lactamase. Pretreatment of donor and recipient strains with the antibiotic clearly increased the frequency of transfer of Tn916 from S. faecalis to B. anthracis. We did not

investigate the mechanism by which the antibiotic augmented transfer of the transposon, however it was previously found (P. A. W. Martin, Abstr. Annu. Meet.,13th International Congress of Microbiology, 1982, p. 47) that cell wall-active antibiotics such as ampicillin and nafcillin increased the rate of transfer in matings on filter membranes of plasmid pAMØ1 from S. faecalis to B. thuringiensis.

It was not surprising that S. faecalis strain CG11Ø transferred Tn916 to B. anthracis at a much higher frequency, over 5ØØ-fold, than S. faecalis strain DS16C1. Gawron-Burke and Clewell (11) reported a similar, enhanced conjugation frequency when strain CG11Ø was used as a donor strain as compared with S. faecalis strains DS16C3 and CG13Ø. The authors suggested (11) that DNA sequences near Tn916 may affect the rate at which the transposon is donated. It was also not unexpected that mating B. anthracis strains VNR-1-tet-1 and UM23-1 in broth yielded no transconjugants, since transfer of Tn916 in broth between streptococci has never been reported.

Hoiseth and Stocker (15) used transposon Tn1Ø mutagenesis to obtain aro- mutants of Salmonella typhimurium, whose growth required phenylalanine, tyrosine, and tryptophan (as well as 2,3-dihydroxybenzoate [DHB] and p-aminobenzoate [PAB] under very stringent nutritional conditions). These aro- mutants were reduced in virulence and were effective as live vaccines in experimental animals. As a result of their inability to synthesize DHB and PAB or to obtain them in the host, infections by the aro- strains were self-limiting. It was the intent of our studies similarly to generate B. anthracis strains which would produce PA and other

anthrax toxin antigens, but would be safer in the immunized host than the current Sterne veterinary vaccine strain. The frequency of excision of Tn916 from the B. anthracis chromosome in the absence of tetracycline and the rates of reversion to prototrophy of the B. anthracis auxotrophic mutants are unknown. In streptococci, however, the frequency of excision and transposition within the cell appears to be related to the frequency of excision and transfer to another cell (11). Franke and Clewell estimated the frequency of transposition of Tn916 in S. faecalis to be approximately 10^{-5} (10). In E. coli Tn916 may excise precisely from DNA, leaving an intact functional gene (12). Thus, some in vivo revertants to prototrophy might be expected after immunization with the two B. anthracis Phe^-, Tyr^-, Trp^- strains isolated in these studies. Nevertheless, the strains should be substantially more attenuated than the nonencapsulated Sterne strain, and they will be tested in experimental animals as prototype, live, vaccine candidates against virulent anthrax spore challenge.

It is clear from these data that Tn916 inserts at different loci in the B. anthracis DNA and that it can be used to obtain specific mutants. It is not clear, however, whether Tn916 inserts preferentially into certain DNA sites. The relative frequency of appearance of Phe^- mutants compared to that of Phe^-, Tyr^-, Trp^- mutants suggests that such may be the case. Further hybridization studies with the various mutants will be required to resolve this question. Other investigations will be conducted to determine the utility of the transposon as a tool for investigating the molecular biology of the bacillus as well as a means of generating specifically

attenuated strains for vaccine studies.

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ADDENDUM

The authors have learned since this manuscript was submitted for publication that J. G. Naglich, R. E. Andrews, Jr., and P. A. Pattee, Iowa State University, have transferred Tn*916* from *S. faecalis* to *Bacillus thuringiensis* by mating on membrane filters (R. E. Andrews, Jr., personal communication). Their investigations and those reported here confirm the ability of *S. faecalis* to transfer Tn*916* to *Bacillus* species where the Tc^r phenotype is then expressed.

LITERATURE CITED

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549-552.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. **7**:1513-1523.
3. **Brewer, C. R., W. G. McCullough, R. C. Mills, W. G. Roessler, and E. J. Herbst.** 1946. Application of nutritional studies for development of practical culture media for Bacillus anthracis. Arch. Biochem. **10**:77-80.
4. **Christie, P. J., R. Z. Korman, S. A. Zahler, J. C. Adsit, and G. M. Dunny.** 1987. Two conjugation systems associated with Streptococcus faecalis plasmid pCF10: Identification of a conjugative transposon that transfers between S. faecalis and Bacillus subtilis. J. Bacteriol. **169**:2529-2536.
5. **Clewell, D. B., G. F. Fitzgerald, L. Dempsey, L. E. Pearce, F. Y. An, B. A. White, Y. Yagi, and C. Gawron-Burke.** 1985. Streptococcal conjugation: plasmids, sex pheromones, and conjugative transposons, p. 194-203. In S. Mergenhagen and B. Rosan (ed.), Molecular basis of oral microbial adhesion. American Society for Microbiology, Washington, D. C.
6. **Clewell, D. B., and C. Gawron-Burke.** 1986. Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Annu. Rev. Microbiol. **40**:635-639.

7. **Clewell, D. B., P. K. Tomisch, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. J. Bacteriol. **152**:122Ø-123Ø.

8. **Dybvig, K., and G. H. Cassell.** 1987. Transposition of gram-positive transposon Tn916 in Achdleplasma laidlawii and Mycoplasma pulmonis. Science **235**:1392-1394.

9. **Franke, A. E., and D. B. Clewell.** 198Ø. Evidence for conjugal transfer of a Streptococcus faecalis transposon (Tn916) from a chromosomal site in the absence of plasmid DNA. Cold Spring Harbor Symp. Quant. Biol. **45**:77-8Ø.

1Ø. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn916) in Streptococcus faecalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145**:494-5Ø2.

11. **Gawron-Burke, C., and D. B. Clewell.** 1982. A transposon in Streptococcus faecalis with fertility properties. Nature (London) **3ØØ**:281-284.

12. **Gawron-Burke, C., and D. B. Clewell.** 1984. Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn916 in Escherichia coli: strategy for targeting and cloning of genes from the gram-positive bacteria. J. Bacteriol. **159**:214-221.

13. **Gonzalez, J. M., Jr., B. J. Brown, and B. C. Carlton.** 1982. Transfer of Bacillus thuringiensis plasmids coding for δ-endotoxin among strains of B. thuringiensis and B. cereus. Proc. Natl. Acad. Sci. USA **79**:6951-6955.

21. **Lauer, G., R. Pastrana, J. Sherley, and M. Ptashne.** 1981. Construction of overproducers of the bacteriophage 434 repressor and cro proteins. J. Mol. Appl. Gen. 1:139-147.

22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

23. **Ristroph, J. D., and B. E. Ivins.** 1983. Elaboration of Bacillus anthracis antigens in a new, defined culture medium. Infect. Immun. 39:483-486.

24. **Roberts, M. C., and G. E. Kenny.** 1987. Conjugal transfer of transposon Tn916 from Streptococcus faecalis to Mycoplasma hominis. J. Bacteriol. **(In press, August, 1987).**

25. **Schleif, R. F., and P. C. Wensink.** 1981. Practical methods in molecular biology, p. 201. Springer-Verlag, New York.

26. **Silhavy, T. J., M. L. Berman, and L. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

27. **Tomisch, P. K., F. Y. An, S. P. Damle, and D. B. Clewell.** 1979. Plasmid-related transmissibility and multiple drug resistance in Streptococcus faecalis subsp. zymogenes strain DS16. Antimicrob. Agents Chemother. 15:828-830.

28. **Tsao, S. G. S., C. F. Brunk, and R. E. Pearlman.** 1983. Hybridization of nucleic acids directly in agarose gels. Anal. Biochem. 131:365-372.

14. **Green, B. D., L. Battisti, T. M. Koehler, C. B., and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in Bacillus anthracis. Infect. Immun. **49**:291-297.

15. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature (London) **291**:238-239.

16. **Inamine, J. M., and V. Burdett.** 1982. Genetic and physical characterization of two chromosomal resistance determinants of Streptococcus agalactiae, p. 170-173. In D. Schlessinger (ed.), Microbiology-1982. American Society for Microbiology, Washington, D. C.

17. **Ivins, B. E., and S. L. Welkos.** 1986. Cloning and expression of the Bacillus anthracis protective antigen gene in Bacillus subtilis. Infect. Immun. **54**:537-542.

18. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. J. Bacteriol. 117:360-372.

19. **Jones, J. M., S. C. Yost, and P. A. Pattee.** 1987. Transfer of the tetracycline resistance transposon Tn916 from Streptococcus faecalis to Staphylococcus aureus and identification of some insertion sites in the staphylococcal chromosome. J. Bacteriol. **169**:2121-2131.

20. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucl. Acid Res. 7:1541-1552.

Table 1. Bacterial strains used

Strain	Chromosome[a] markers	Plasmids[b]	Source or derivation
S. faecalis DS16C1	tet (Tn916)	pAD2 (Km^r Sm^r Em^r [Tn917])	D. Clewell; 7, 27
S. faecalis JH2-2	rif fus	None	D. Clewell; 18
S. faecalis CG11Ø	rif fus tet (Tn916)	None	D. Clewell; 1Ø, 11; S. faecalis DS16C3 S. faecalis JH2-2
Escherichia coli DH1	F recA1 endA1 gyr96 thi-1 hsdR17 supE44	pJI3 (Tc^r [tetM])	16; pACYC177 carrying tetM on a 5-Kb HinCII fragment
E. coli CG12Ø	same as E. coli DH1	pAM12Ø (Ap^r Tc^r [Tn916])	D. Clewell; 12
E. coli CG12ØLT	same as E. coli DH1	pAM12ØLT (Ap^r)	D. Clewell; 12
B. anthracis VNR-1	None	pXO1 (Tox$^+$)	14; cured of pXO2 by novobiocin
B. anthracis UM23-1	str	pXO1 (Tox$^+$)	C. B. Thorne
B. anthracis VNR-1-tet-1	tet (Tn916)	pXO1 (Tox$^+$)	This study; S. faecalis DS16C1 X B. anthracis VNR-1
B. anthracis VNR-1-tet-2	tet (Tn916)	pXO1 (Tox$^+$)	This study; S. faecalis CG11Ø X B. anthracis VNR-1
B. anthracis VNR-1-tet-3	tet (Tn916)	pXO1 (Tox$^+$)	This study; S. faecalis CG11Ø X B. anthracis VNR-1
B. anthracis VNR-1-tet-4	tet (Tn916)	pXO1 (Tox$^+$)	This study; S. faecalis CG11Ø X B. anthracis VNR-1
B. anthracis VNR-1-tet-B	str tet (Tn916) phe	pXO1 (Tox$^+$)	This study; B. anthracis VNR-1-tet-1 X B. anthracis UM23-1

B. anthracis VNR-1-tet-C	str tet (Tn916) phe tyr trp	pXO1 (Tox+)	This study; B. anthracis VNR-1-tet-1 X B. anthracis UM23-1
B. subtilis BST1	spoOA(Delta)677	pUB110 (Km^r)	17; B. subtilis BGSC 1S53 containing pUB110
B. subtilis KT1	spoOA(Delta)677 tet (Tn916)	pUB110 (Km^r)	This study; B. anthracis VNR-1-tet-1 X B. subtilis BST1

[b] Antibiotic resistance markers: rif = rifampicin; fus = fusidic acid; tet = tetracycline; str = streptomycin.

[a] Km = kanamycin; Sm = streptomycin; Em = erythromycin; Tc = tetracycline; Ap = ampicillin.

TABLE 2. Filter mating transfer of tetracycline resistance[a]

Donor: Recipient	No. Replicate Samples	Frequency[b] ±SEM[c]
S. faecalis DS16C1: B. anthracis VNR-1[d]	8	1.6 X 10^{-7} ±0.5 X 10^{-7}
B. anthracis VNR-1-tet-1: B. anthracis UM23-1[e]	4	2.1 X 10^{-4} ±0.8 X 10^{-4}
B. anthracis VNR-1-tet-1: B. subtilis BST1[f]	3	5.8 X 10^{-6} ±2.6 X 10^{-6}
S. faecalis CG110: B. anthracis VNR-1[d]	1	9.3 X 10^{-5}[g]

[a] For experimental details, see text.

[b] The frequency was expressed as the number of Tc^r transconjugants divided by the number of Tc^r donor cells at the end of mating.

[c] Standard error of the mean.

[d] Selection medium = RA (to select against donor) with tetracycline (to select against recipient).

[e] Selection medium = BHIA with streptomycin and tetracycline.

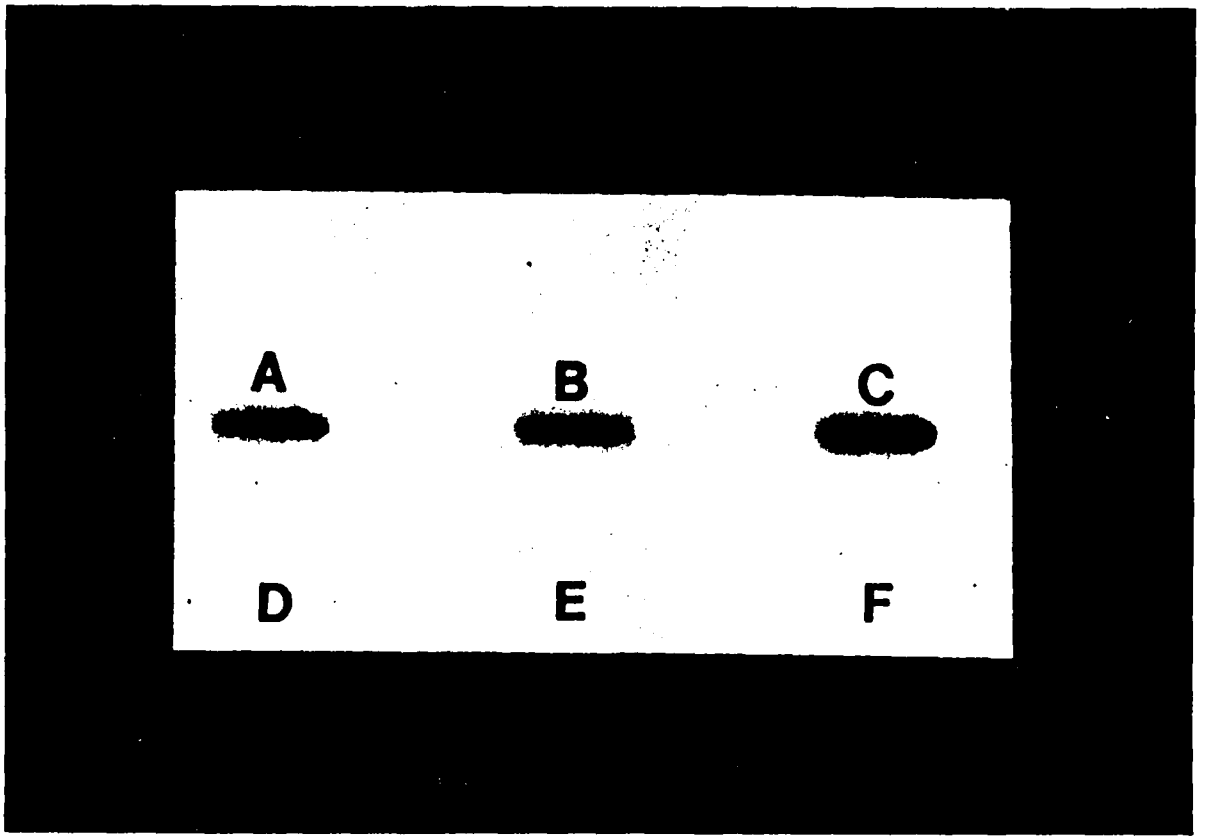
[f] Selection medium = BHIA with kanamycin and tetracycline.

[h] No SEM was calculated.

FIGURE LEGENDS

FIG. 1 - "Slot Blot" hybridization of total-cell DNA with a 5-kb probe for the tetM determinant of Tn916. Total-cell DNA (5ØØ μg/sample) from Tc^r and Tc^s strains was transferred to nitrocellulose membrane and hybridized to the ^{32}P-labeled probe. A, B. subtilis KT1; B, S. faecalis DS16C1; C, B. anthracis VNR-1-tet-1; D, B. subtilis BST1; E, S. faecalis JH2-2; F, B. anthracis VNR-1.

FIG. 2 - Hybridization of digested, total B. anthracis cell DNA with ^{32}-P-labeled lambda DNA and pAM12Ø DNA. Total-cell DNA was digested with HindIII enzyme, subjected to electrophoresis in Ø.8% agarose, and probed (see text). Lanes: A, lambda digested with HindIII and EcoRI; B, B. anthracis VNR-1-tet-4; C, B. anthracis VNR-1-tet-3; D, B. anthracis VNR-1-tet-2; E, B. anthracis VNR-1-tet-1; F, B. anthracis tet C; G, B. anthracis tet B; H, B. anthracis VNR-1; I, pAM12Ø (undigested). The total-cell DNA digests did not hybridize with a pAM12ØLT probe.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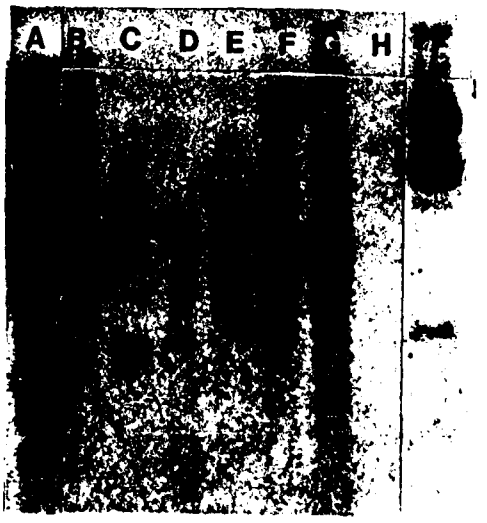
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